

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
4 January 2001 (04.01.2001)

PCT

(10) International Publication Number
WO 01/01116 A1

(51) International Patent Classification⁷:

G01N 21/64

(81) Designated States (*national*): AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(21) International Application Number:

PCT/US00/16286

(22) International Filing Date:

14 June 2000 (14.06.2000)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

60/141,058 25 June 1999 (25.06.1999) US
09/374,951 16 August 1999 (16.08.1999) US

(71) Applicant: NOVUS INTERNATIONAL, INC. [US/US];
530 Maryville Centre Drive, St. Louis, MO 63141 (US).

(72) Inventors: ANDREWS, John, T.; 530 Maryville Centre Drive, St. Louis, MO 63141 (US). SHERMER, William, D.; 530 Maryville Centre Drive, St. Louis, MO 63141 (US). GIESEN, Andrew, F.; 530 Maryville Centre Drive, St. Louis, MO 63141 (US).

(74) Agents: ROEDEL, John, K., Jr. et al.; Senniger, Powers, Leavitt & Roedel, 16th floor, One Metropolitan Square, St. Louis, MO 63102 (US).

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

- With international search report.
- Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.



WO 01/01116 A1

(54) Title: SEMI-QUANTITATIVE FLUOROMETRIC METHOD FOR DETECTION OF ETHOXYQUIN

(57) Abstract: A method of semi-quantitatively measuring levels of ethoxyquin within samples of vegetable or animal products or vitamin products in the field by extracting ethoxyquin into a solution and measuring the solution in a fluorometer equipped with a long-wavelength ultraviolet light. The solution contains water, salt, a water-immiscible organic solvent, and optionally a polar solvent. The concentration of ethoxyquin is estimated by comparing the fluorometer's response to the sample solution with a calibration chart derived from known concentrations of ethoxyquin.

SEMI-QUANTITATIVE FLUOROMETRIC METHOD
FOR DETECTION OF ETHOXYQUIN

BACKGROUND OF INVENTION

This application claims priority to U.S. provisional
5 patent application Ser. No. 60/141,058 filed June 25,
1999.

Field of the Invention

The present invention generally relates to a field test for ethoxyquin with minimal sample preparation and 10 quick, on-site results that can be readily performed by shipping, quality control and/or receiving personnel verifying ethoxyquin to semi-quantitatively determine concentrations in vegetable or animal products.

Description of Related Art

15 Animal feed ingredients such as fats and vitamins bring a value to animal diets. Fats in animal feed supply energy for both maintenance and growth of the animals. They provide essential fatty acids for the synthesis of prostaglandins. They also facilitate the 20 adsorption of fat soluble vitamins and enhance flavor. In addition, fats reduce dust in feed mills during the manufacture of animal feed and at grow-out houses.

Oxidation can significantly reduce many of the benefits provided by the addition of fats and vitamins 25 and can also introduce damaging by-products. For example, oxidation consumes energy in the fat and that energy is then unavailable to an animal. Oxidation begins when a carbon-hydrogen bond in a fat or vitamin breaks to produce a chemical species called a free 30 radical. This free radical reacts with the oxygen in air

to produce compounds called peroxides. Peroxides are unstable products and continue to react further to give a wide variety of by-products including aldehydes, ketones, alcohols, esters, acids and polymers.

5 Some of the by-products from oxidation, especially the aldehydes, can be toxic to the animal. Fat soluble vitamins can be destroyed leading to deficiency syndromes such as steatitis and encephalomalacia. Damage can be seen even at the cellular level due to the reaction with
10 peroxides or the toxicity of the aldehydes produced as by-products of the oxidation process. As a consequence of that oxidation, an animal will not reach its full potential and the net result to the producer is poorer financial return due to lower body weight and poorer feed
15 conversion. These problems can be minimized with good quality control which begins with monitoring the quality of the ingredients received at rendering plants and feed mills.

In 1988, Cabel and Waldroup reported on a study with
20 male broilers fed four diets containing specified levels of oxidized fat. The control diet was prepared with unoxidized fats. Cabel, M.C., et al., 1988, Effects of Ethoxyquin Feed Preservative and Peroxide Level on Broiler Performance, Poultry Science, 67:1725-1730. The
25 other three diets were prepared from oxidized fats and formulated to contain 2, 4, and 7 meq. of peroxides per kg of feed, respectively. A trend to poorer body weight was observed as the level of peroxides increased. The birds fed the diets containing 7 meq peroxides/kg of feed
30 had significantly lower body weight than the birds fed the control diet. There was also a trend to poorer feed conversion as the peroxide content of the diets increased. The negative effects of oxidation were

mitigated by the inclusion of ethoxyquin in the diets prepared with oxidized fat.

A similar study was conducted by Dr. Schang and coworkers using rancid meat meal with identical results.

5 These studies confirm that feeding oxidized fats can cost the grower money. Schang, M.J., et al., 1987, Quality of Ingredients and Their Effect on Growth: Rancidity, 10th LATAM Poultry Congress, Buenos Aires, Argentina, 29 September - 2 October, 221.

10 A three-week feeding study was conducted by Dibner et al. using oxidized fat to try to understand what were the underlying reasons that produced the poorer body weight and feed conversions reported by Cabel et al.

(1988) and by Schang et al. (1987). Dibner, J.J., et 15 al., 1996, Feeding of Oxidized Fats to Broilers and Swine: Effects on Enterocyte Turnover, Hepatocyte Proliferation and the Gut Associated Lymphoid Tissue, Animal Feed Science and Technology, 62:1-13. Four diets were used in the study. Two of these included fresh fat

20 with and without ethoxyquin. The other two diets were prepared with or without ethoxyquin using 4% fat that had been oxidized to 100 meq of peroxides. This produced diets that had 4 meq peroxides per kg of feed. The same effects of reduced body weight and reduced feed

25 conversion were seen in this study as seen by Cabel and by Schang. The birds fed the diets containing oxidized fat plus 125 ppm of ethoxyquin had significantly better feed to gain than those birds consuming the diets prepared with the oxidized fat without ethoxyquin. The

30 birds on the control diets performed better than those birds consuming oxidized fat. But even here, there was a trend to better performance in the diet with ethoxyquin even when those diets were prepared with fresh fat. This was evident as early as 14 days with a significantly

better feed to gain for those birds consuming the fresh fat with ethoxyquin compared with those birds consuming either of the two diets prepared with the oxidized fat. An intermediate level of performance was observed for 5 those birds consuming the control diet, but without the added ethoxyquin. Of particular interest was the observation that the trends could be seen already within seven days, although the differences were not yet significant at that time.

10 The poorer body weight and feed conversion are the external effects that can be seen by the grower. Several factors were observed in the study at the cellular level that could contribute to those measurable results. First, the pattern of concentrations of red blood cells 15 was exactly the same as the body weight. The highest concentration was in the birds fed the fresh fat plus ethoxyquin and the lowest concentration was in the birds fed the diets prepared from oxidized fat without added ethoxyquin. Second, consuming the diets prepared with 20 oxidized fat and no ethoxyquin caused a transient reduction of the Lactobacilli in the microflora of the gut. With the reduced Lactobacilli, the *E. coli* grew heavily. Third, the birds fed the diets with the oxidized fat had measurably increased liver cell and 25 intestinal epithelial cell turnover showing the impact of the peroxides and the toxic by-products from oxidation in these tissues. Finally, there was a reduced level of IgA in the gut of the birds fed the oxidized fat suggestive of reduced immune response.

30 Oxidation can be controlled through the use of an antioxidant such as ethoxyquin. Ethoxyquin, or 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline, is a chemical that is used by rendering plants, animal processing plants, and feed mills as a preservative or stabilizer in a

variety of animal and vegetable products. Ethoxyquin is a free radical trap. It can intercept the free radicals to stop the formation of peroxides and prevent the formation of the other by-products. This enables the ethoxyquin to 5 control the oxidation process, limit the damage from the free radicals and limit the development of the toxic by-products.

Ethoxyquin is used in a variety of animal feed ingredients and products such as animal and vegetable 10 products, vitamins and vitamin premixes to preserve the materials and prevent oxidative degeneration. The animal and vegetable products produced by rendering plants include such materials as poultry meal, meat and bone meal, fish meal, poultry fat, animal fat, tallow, lard, 15 yellow grease made from animal or vegetable oils and fats, and other by-product meals (hereinafter referred to as "rendered products"). The rendering plants may add ethoxyquin to their products at any period during the processing of the animal or vegetable materials. The 20 rendered products are then shipped to feed mills for incorporation into animal feeds.

Animal processing plants also add ethoxyquin to waste animal oils and fats that are removed from machines, floors, and waste water in order to comply with 25 environmental regulations. These waste animal oils and fats are termed diffused air floatation (DAF) sludge. The DAF sludge is then shipped to rendering plants as a raw material to also be incorporated with other raw materials for processing into rendered products.

30 Vitamins and vitamin premixes (hereinafter referred to as "vitamin products") that are added to animal feed also contain ethoxyquin as an ingredient to stabilize the vitamin products.

The problems associated with oxidation of fats can therefore be minimized by ensuring the presence of ethoxyquin in the rendered products, DAF sludge, or vitamin products and by monitoring the quality of the 5 ingredients received at rendering plants and feed mills. Stabilization of the high fat ingredients is a key step in maintaining quality.

As shipments of DAF sludge are received by rendering plants or rendered products are received by feed mills, 10 the only way to currently determine whether the products contain ethoxyquin is to conduct a chemical analysis on a sample. This requires that a sample be analyzed at either the receiving plant's in-house laboratory or to ship a sample to an off-site analytical laboratory to 15 determine the levels of ethoxyquin using known analytical methods. This process is time consuming, requiring overnight analysis for in-house laboratories or several days or weeks for shipping and analysis at off-site laboratories.

20 Rendering plants, feed mills, and the shipping trucks delivering the materials to these plants do not have time to wait overnight or a week or two to receive analytical results before accepting the shipped products or having the products mixed in feed. A need has 25 therefore been identified to develop a screening tool that can be used to quickly determine whether rendered products, DAF sludge, or vitamin products are stabilized with ethoxyquin. Such a method is needed as only a screening tool to roughly estimate the ethoxyquin levels 30 present in the shipped materials rather than provide precise analytical quantification of the ethoxyquin levels in the materials.

While the benefits of ethoxyquin have been long known, no method has been available that would allow

rendering plant or feed mill personnel to test for an antioxidant in the rendered products, DAF sludge, or vitamin products before they could be unloaded from the truck or railroad car into the storage bin. Three qualities increase an ethoxyquin detection method's usefulness to a rendering plant, processing plant, or feed mill. The first quality is the ease in which the method may be performed. A method is most useful if it can be conducted by an employee without technical training. The second is the time required to obtain the results of the detection method. Such a method is most useful if the time is short enough that a truck driver would wait for the analysis to be completed before unloading the truck and putting the ingredient into the storage tanks. The final quality is the expense of the method. The method is most useful if it is inexpensive. If it is too expensive, the rendering plants and feed mills may not spend the money to do it.

20

SUMMARY OF INVENTION

Accordingly, it is an object of the present invention to provide a semi-quantitative method in which the presence of ethoxyquin is able to be approximated in a material of animal or vegetable origin, or vitamin product processed by rendering plants, animal processing plants, and manufacturers of feed vitamins.

Briefly, therefore, the present invention is directed to a method in which a sample of material of animal or vegetable origin, or vitamin product is combined with a solvent to form an extract. A mixture is formed from the extract, a salt, water, and water-immiscible organic solvent. The water-immiscible organic solvent is separated from the mixture and the ethoxyquin

concentration is measured using a fluorometer equipped with an ultraviolet light.

In another aspect, the invention is directed to a method in which a sample of material of animal or

5 vegetable origin, or vitamin product is mixed with a polar solvent forming a first extraction mixture. A second extraction mixture is formed from a salt, water, a water-immiscible organic solvent, and the polar solvent from the first extraction mixture. The water-immiscible

10 organic solvent is separated from the polar solvent and the ethoxyquin concentration is measured using a fluorometer equipped with an ultraviolet light.

In another aspect, the invention is directed to a method in which a sample of material of animal or

15 vegetable origin, or vitamin product is combined with a polar solvent forming a first extraction mixture. A second extraction mixture is formed from a salt, water, a water-immiscible organic solvent, and an absence of a polar solvent other than the polar solvent from the first

20 extraction mixture. The water-immiscible organic solvent is separated from the polar solvent and the ethoxyquin concentration is measured using a fluorometer equipped with an ultraviolet light.

In a further aspect, the invention is directed to a

25 method in which a sample of material of animal or vegetable origin, or vitamin product is combined with a polar solvent forming a first extraction mixture without grinding the material to a desired size. A second extraction mixture is formed from a salt, water, a water-

30 immiscible organic solvent, and the polar solvent from the first extraction mixture. The water-immiscible organic solvent is separated from the polar solvent and the ethoxyquin concentration is measured using a fluorometer equipped with an ultraviolet light.

In a further aspect, the invention is directed to a method in which a sample of material of animal or vegetable origin, or vitamin product is combined with a polar solvent forming a first extraction mixture without 5 measuring the mass of the sample within 0.1 grams. A second extraction mixture is formed from a salt, water, a water-immiscible organic solvent, and the polar solvent from the first extraction mixture. The water-immiscible organic solvent is separated from the polar solvent and 10 the ethoxyquin concentration is measured using a fluorometer equipped with an ultraviolet light.

In a further aspect, the invention is directed to a method in which a sample of material of animal or vegetable origin, or vitamin product is combined with a 15 water-immiscible organic solvent forming a first extraction mixture. A second mixture is formed from a salt, water, and the water-immiscible organic solvent from the first extraction mixture. The water-immiscible organic solvent is then separated from the mixture and 20 the ethoxyquin concentration is measured using a fluorometer equipped with an ultraviolet light.

In still a further aspect, the invention is directed to a method in which a sample of material of animal or vegetable origin, or vitamin product is combined with a 25 water-immiscible organic solvent, a salt, and water. The water-immiscible organic solvent is separated from the mixture and the ethoxyquin concentration is measured using a fluorometer equipped with an ultraviolet light.

Other features of the present invention will be in 30 part apparent to those skilled in the art and in part pointed out in the detailed description provided below.

BRIEF DESCRIPTION OF THE DRAWING

Fig. 1 is a fluorometer calibration chart correlating the fluorometer reading to an estimated concentration of ethoxyquin.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

5 Rendering plant and feed mill personnel would benefit from having an analytical method for ethoxyquin that can quickly determine if ethoxyquin was added to the incoming ingredients before a truck or railroad car containing rendered products, DAF sludge, or vitamin 10 products is unloaded. This represents "Real Time" analysis:

The present invention ("the semi-quantitative method") has been developed to semi-quantitatively estimate the level of ethoxyquin in rendered products, 15 DAF sludge, and vitamin products. This test can be run frequently because it is easy, inexpensive and rapid enough to be used by personnel such as rendering plant and feed mill employees. More frequent analyses will provide more control over the suppliers and better 20 quality control of feed ingredients and the feed produced from them. This should ultimately lead to better animal performance (e.g. less feed is required to increase animal body weight) and better profits.

Ethoxyquin has a property of fluorescing when placed 25 under an ultraviolet light, emitting a wavelength that can be optimally detected by a fluorometer around 405 nm.

Sometimes it is desirable to have an estimate of how much ethoxyquin is actually present. Using the semi-quantitative method, one can determine if a sample 30 contains ethoxyquin within 15% to 30% for a given source of material from an animal or vegetable origin, vitamins, and vitamin premixes. If a scale is used to weigh the

sample, repeat analyses of the same sample or samples from the same supplier can be accurate within 15% of the actual amount of ethoxyquin without doing all of the work required by the official AOAC procedure for quantifying 5 ethoxyquin. The semi-quantitative method can provide a good estimate which is accurate within 30% of the amount of ethoxyquin present even though the sample size is simply measured using a spoon and including the variation in sample composition for the rendered products, DAF, and 10 vitamin products from different sources.

Rendering plant products, DAF sludge, and vitamin products can therefore be tested for the presence of ethoxyquin by extracting any ethoxyquin present in the product into solution. This can be achieved by mixing 15 rendered products, DAF, or vitamin products with a single portion of solvent for a period of time and then allowing the mixture to settle. The weight of the sample does not have to be precisely measured because the semi-quantitative method is not designed to provide an 20 accurate quantitative number. A large spoon such as a tablespoon or soup spoon can be used to measure the sample (e.g. about 5 g or 15 mL). It is therefore unnecessary to grind the sample to a desired size or measure the mass of the sample within 0.1 grams prior to 25 mixing the sample with a solvent.

About 1.5 parts of sample by volume (e.g. about 5 g or 15 mL) is placed in a beaker or a similar container with about 5 parts solvent (e.g. about 15 mL sample is mixed with about 50 mL solvent). The solvent is 30 preferably a polar solvent, although a water-immiscible organic solvent may also be used. Examples of polar solvents that may be used include, but are not limited to, methanol, ethanol, isopropyl alcohol, acetonitrile, and acetic acid. Examples of water-immiscible organic

solvents include, but are not limited to, petroleum ether, toluene, chloroform, and alkanes (such as pentanes, hexanes, octanes, iso-octane, and cyclohexane). The sample is preferably shaken or stirred for about 2 minutes. Mechanical stirring with a magnetic stirrer is convenient to use, or the container can be sealed and shaken by hand.

A fraction of the solvent portion of the mixture is then removed (e.g. approximately 1 part or about 10 mL). 10 The sample residue need not be extracted further with fresh portions of a solvent. The removed fraction of the solvent is then poured into a disposable plastic tube or similar container and thoroughly mixed together with water (e.g. approximately 1 part solvent by volume per 3 parts water or about 10 mL solvent per 30 mL water), a 15 sufficient quantity of salt to give a 1.5% to 10% salt concentration when mixed with the water, and a water-immiscible organic solvent (e.g. approximately 1 part or about 10 mL of water-immiscible organic solvent). Salts 20 which may be used are inorganic salts, examples of which include, but are not limited to, salts of alkali metal and alkali earth metal such as sodium chloride, potassium chloride, magnesium sulfate, and any monovalent or divalent inorganic salt. Examples of water-immiscible 25 organic solvents include, but are not limited to, petroleum ether, toluene, chloroform, and alkanes (such as pentanes, hexanes, octanes, iso-octane, cyclohexane).

The tube is preferably sealed and shaken for about 15 seconds. The salt and water act to break up any 30 emulsion between the water-miscible layer and water-immiscible organic solvent layer by providing an ionic source to separate the layers. If ethoxyquin is present in the sample, it will be pulled into the water-immiscible organic solvent.

The water-immiscible organic solvent layer is then allowed to separate from the mixture into its own layer after the mixture has been shaken. One portion of the top layer of the water-immiscible organic solvent is then

5 placed in a volumetric flask or similar container and diluted to ten times the volume of the portion (e.g. about 1 mL is diluted to 10 mL) with additional water-immiscible organic solvent. The remaining mixture need not be further extracted with additional fresh portions

10 of water-immiscible organic solvent.

Sufficient volume of the diluted water-immiscible organic solvent solution is used to fill the sample cell for the fluorometer. This solution is measured with a calibrated fluorometer and the fluorescence value is

15 read. If the concentration of ethoxyquin present in the original sample was above approximately 50 ppm, the solution containing ethoxyquin will have a measurable emission wavelength between 395-430 nm, preferably around 405 nm, when measured in a fluorometer with an excitation

20 wavelength between 355-375 nm, preferably around 360 nm. By comparing the instrumental response to a calibration chart, an example of which is illustrated in Fig. 1, a semi-quantitative concentration can be estimated.

Positive results for the semi-quantitative test for

25 ethoxyquin can be estimated within 15% to 30% with a detection level of 50 ppm and sometimes as low as 25 ppm, depending on variations in sources of tested materials.

The preferred embodiment is to mix about 1.5 parts of the sample by volume with about 5 parts methanol (e.g. 30 approximately 15 mL or 1 tablespoon sample is mixed with about 50 mL methanol) to form a first extraction mixture. The sample is shaken or stirred for about 2 minutes. A fraction of the methanol portion of the mixture is then removed (e.g. approximately 1 part or about 10 mL). The

removed methanol fraction from the first extraction mixture is combined with 3 parts water (e.g. approximately 1 part solvent by volume per 3 parts water or about 10 mL solvent per 30 mL water), a quantity of 5 sodium chloride to give a 1.5% to 10% sodium chloride concentration when mixed with the water, and approximately 1 part petroleum ether (e.g. about 10 mL of petroleum ether) to form a second extraction mixture. The tube is sealed and shaken for about 15 seconds. The 10 petroleum ether layer is then allowed to separate from the second extraction mixture into its own layer after the mixture has been shaken. One portion of the petroleum ether is then placed in a volumetric flask or similar container and diluted to ten times the volume of 15 the portion (e.g. about 1 mL is diluted to 10 mL) with additional petroleum ether. The remaining mixture need not be further extracted with additional fresh portions of petroleum ether. Sufficient volume of the diluted petroleum ether solution is used to fill the sample cell 20 for the fluorometer. This solution is measured in a fluorometer with an excitation wavelength between 355-375 nm, most preferably around 360 nm. If the concentration of ethoxyquin present in the original sample was above approximately 50 ppm, the solution containing ethoxyquin 25 will have a measurable emission wavelength between 395-430 nm, most preferably around 405 nm. The semi-quantitative concentration can be estimated by comparing the fluorescence value to a calibration chart, an example of which is illustrated in Fig. 1.

30 The test may be simplified without using a polar solvent. Similar to the steps above, a large spoon such as a tablespoon or soup spoon can be used to measure the sample. About 1.5 parts of sample by volume (e.g. about 5 g or 15 mL) is placed in a beaker or a similar

container with about 5 parts of a water-immiscible organic solvent (e.g. about 15 mL sample is mixed with about 50 mL water-immiscible organic solvent) to form a first extraction mixture. The first extraction mixture 5 container and is preferably shaken or stirred for about 2 minutes. A fraction of the solvent portion of the mixture is then removed (e.g. approximately 1 part or about 10 mL). A second extraction mixture is formed by combining the removed fraction of the water-immiscible 10 organic solvent, water (e.g. approximately 1 part water-immiscible organic solvent by volume per 3 parts water or about 10 mL water-immiscible organic solvent per 30 mL water), and a sufficient quantity of salt to give a 1.5% to 10% salt concentration when mixed with the water. The 15 second extraction mixture is shaken or stirred for about 15 seconds. The liquid layers are allowed to separate. One portion of the top layer of the water-immiscible organic solvent is then placed in a volumetric flask or similar container and diluted to ten times the volume 20 (e.g. about 1 mL is diluted to 10 mL) with additional water-immiscible organic solvent. The remaining mixture need not be further extracted with additional fresh portions of water-immiscible organic solvent. A sufficient volume of the diluted water-immiscible organic 25 solvent solution is used to fill the sample cell for the fluorometer.

In the final step, the sample cell is placed into the fluorometer and the fluorescence value is read. The analyst compares this number with a fluorometer 30 calibration chart, similar to that illustrated in Fig. 1, and determines the estimated ppm of ethoxyquin.

The test may be simplified even further by preparing an extraction mixture consisting of about 3 parts water (e.g. about 30 mL), a sufficient quantity of

salt to give a 1.5% to 10% salt concentration when mixed with the water, about 1.5 parts of the sample (e.g. about 5 g or 15 mL), and about 5 parts water-immiscible organic solvent (e.g. approximately 50 mL). The extraction
5 mixture is preferably shaken or stirred for about 2 minutes. The liquid layers are allowed to separate. One portion of the top layer of the water-immiscible organic solvent is then placed in a volumetric flask or similar container and diluted to ten times the volume of the
10 portion (e.g. about 1 mL is diluted to 10 mL) with additional water-immiscible organic solvent. The remaining mixture need not be further extracted with additional portions of water-immiscible organic solvent. A sufficient volume of the diluted water-immiscible
15 organic solvent solution is used to fill the sample cell for the fluorometer.

In the final step, the sample cell is placed into the fluorometer and the fluorescence value is read. The analyst compares this number with a fluorometer
20 calibration chart, similar to that illustrated in Figure 1, and determines the estimated ppm of ethoxyquin.

The semi-quantitative method can utilize a variety of solvents and salts to achieve the desired test results. Reagent grade solvents and salts provide the
25 best test results as they lack impurities that can cause interferences in the test.

The hardware necessary to measure the fluorescence of the sample solution is a fluorometer with an excitation filter and emission filter. The excitation
30 and emission filters are selected based upon the excitation wavelength range of 355-375 nm, preferably about 360 nm, and an emission wavelength of 395-430 nm, preferably about 405 nm.

In using the semi-quantitative method for ethoxyquin, rendering plants, processing plants, and feed mills will be able to quickly detect the presence and estimate the level of ethoxyquin in rendered products,

5 DAF sludge, and vitamin products prior to being shipped or received by the plants or mills while they have the products sent out for more precise quantitative analysis.

In this way, the method promotes improved quality of rendered products, DAF sludge, vitamin products and

10 finished feed by confirming in "real time" that rendered products, DAF sludge, and vitamin products contain ethoxyquin when specified. Finally, this method brings added value to ethoxyquin products, permitting the preservative to be tested for continued effectiveness in

15 the materials it is intended to preserve.

The invention method is not designed to provide the same degree of precision as the official method for the measurement of ethoxyquin. The invention method is semi-quantitative. The standard analytical method published

20 by the AOAC should be used if exact levels of ethoxyquin are required. Tables I & II compare the semi-quantitative method and the official AOAC method for detecting ethoxyquin. The semi-quantitative method has a minimum detection level of 50 ppm, and sometimes as low

25 as 25 ppm, depending on the sample source materials; whereas the official method can be used down to levels of 20 ppm.

The real advantage of the semi-quantitative method regards the time and costs of running the tests. The

30 semi-quantitative method takes only 3 to 5 minutes per analysis whereas the AOAC method takes a minimum of 2 hours to complete. Non-technical personnel can easily be trained to run the invention method at rendering plants or feed mills. Typically, the official procedure cannot

be run at rendering plants or feed mills because it requires a trained technician, who is located either in the company's central lab or in a commercial lab. This means that the time required for the AOAC method of 5 analysis may be extended to days while the samples are transported to the lab site.

Therefore, the cost savings are primarily a combination of a reduction in time for an employee to run the test plus a lower cost for items consumed during the 10 analysis. The cost to have a employee at a rendering plant or feed mill run a test in 5 minutes is much less than the cost of a two hour analysis run by a technician in a laboratory. The on-site analysis also eliminates any shipping charges.

TABLE I
Method Comparisons in Commercial Fats

Sample	AOAC Official Method for Ethoxyquin ¹ (ppm)	Semi-Qualitative Test for Ethoxyquin (ppm)
Poultry Fat	250	198
Lard	342	300
Yellow Grease	100	66

¹ AOAC Official Method 963.07, Official Methods of Analysis of AOAC International, 16th Ed. 1998:4.10.02, Chapter 4, p. 43.

TABLE II
Method Comparisons in Commercial By-Product Meals

Sample	AOAC Official Method for Ethoxyquin ¹ (ppm)	Semi-Quantitative Test for Ethoxyquin (ppm)
Poultry Meal	104	100
Poultry Meal	230	200
Meat Meal	143	200
Poultry/Fish Meal	194	250

¹ AOAC Official Method 963.07, Official Methods of Analysis of AOAC International, 16th Ed. 1998:4.10.02, Chapter 4, p. 43.

EXAMPLE 1 - Semi-Quantitative Method for EthoxyquinMaterials

Methanol, petroleum ether and sodium chloride, all commercially available reagent grade chemicals. Magnetic stirrer and stir bars, glass beakers, glass test tubes, and fluorometer (360 nm excitation wavelength filter, 405 nm emission wavelength filter), all commercially available. Deionized or tap water.

Method

Add approximately 5 grams of sample (fat or meal) and 50 mL of methanol to a glass beaker. Stir manually or mechanically (e.g. with a magnetic stirrer) for 2 minutes. Allow solid material to settle.

Decant 10 mL of supernatant to another beaker, add 30 mL of a 1.5% to 10% salt water solution and 10 mL petroleum ether and shake for 15 seconds. Allow mixture to settle undisturbed and separate into polar and water-immiscible organic solvent layers.

Dilute 1 portion top organic layer (petroleum ether) with 9 parts petroleum ether, mix, and transfer to a fluorometric examination tube. Measure the instrumental response on a fluorometer. The approximate concentration of ethoxyquin is estimated by comparing the instrumental response to calibration chart values.

The responses will vary within 15% to 30% depending on variations in sources of sampled products. The invention method has a minimum detection level of 50 ppm, and sometimes as low as 25 ppm, depending on the sample source materials.

EXAMPLE 2 - Qualitative Test for Ethoxyquin Without a Polar Solvent in Two Extraction Mixtures

Materials

Petroleum ether and sodium chloride, all
5 commercially available reagent grade chemicals. Magnetic stirrer and stir bars, glass beakers, glass test tubes, and fluorometer (360 nm excitation wavelength filter, 405 nm emission wavelength filter), all commercially available. Deionized or tap water.

10 Method

Add approximately 5 grams of sample (fat or meal) and 50 mL of petroleum ether to a glass beaker. Stir manually or mechanically (e.g. with a magnetic stirrer) for 2 minutes. Allow solid material to settle.

15 Decant 10 mL of supernatant to another beaker, add 30 mL of a 1.5 to 10% salt water solution and shake for 15 seconds. Allow mixture to settle undisturbed and separate into salt water and water-immiscible organic solvent layers.

20 Dilute 1 portion top water-immiscible organic layer (petroleum ether) with 9 parts petroleum ether, mix, and transfer to a fluorometric examination tube. Measure the instrumental response on a fluorometer. The approximate concentration of ethoxyquin is estimated by comparing the
25 instrumental response to calibration chart values.

The responses will vary within 15-30%, depending on variations in sources of sampled products. The invention method has a minimum detection level of 50 ppm, and sometimes as low as 25 ppm, depending on the sample
30 source materials.

EXAMPLE 3 - Qualitative Test for Ethoxyquin Without a
Polar Solvent in One Extraction Mixture

Materials

Petroleum ether and sodium chloride, all
5 commercially available reagent grade chemicals. Magnetic
stirrer and stir bars, glass beakers, glass test tubes,
and fluorometer (360 nm excitation wavelength filter, 405
nm emission wavelength filter), all commercially
available. Deionized or tap water.

10 Method

Add approximately 5 grams of sample (fat or meal)
and 50 mL of petroleum ether, 30 mL of a 1.5 to 10% salt
water solution to a glass beaker. Stir manually or
mechanically (e.g. with a magnetic stirrer) for 2
15 minutes.

Allow mixture to settle undisturbed and separate
into salt water and water-immiscible organic solvent
layers.

Dilute 1 portion top water-immiscible organic layer
20 (petroleum ether) with 9 parts petroleum ether, mix, and
transfer to a fluorometric examination tube. Measure the
instrumental response on a fluorometer. The approximate
concentration of ethoxyquin is estimated by comparing the
instrumental response to calibration chart values.

25 The responses will vary within 15 to 30%, depending
on variations in sources of sampled products. The
invention method has a minimum detection level of 50 ppm,
and sometimes as low as 25 ppm, depending on the sample
source materials.

30 As various changes could be made in the above method
without departing from the scope of the invention, it is

intended that all matter contained in the above description be interpreted as illustrative and not in a limiting sense.

We Claim:

1. A method for approximating the concentration of ethoxyquin in a material of animal or vegetable origin, or vitamin product comprising:
 - 5 combining the material with a solvent to form an extract;
 - forming a mixture comprising the extract, a salt, water, and a water-immiscible organic solvent;
 - separating water-immiscible organic solvent from the
 - 10 mixture in a single extraction step; and
 - determining the concentration of ethoxyquin in the separated water-immiscible organic solvent using a fluorometer.
2. The method of claim 1 wherein the material of animal or vegetable origin, or vitamin product is selected from the group consisting of poultry meal, meat and bone meal, fish meal, poultry fat, animal fat, tallow, lard, yellow grease made from animal or vegetable oils and fats, by-product meals, vitamins and vitamin premixes, and DAF sludge.
3. The method of claim 1 wherein the water-immiscible organic solvent is selected from the group consisting of petroleum ether, toluene, chloroform, and alkanes.
4. The method of claim 3 wherein the alkanes are selected from the group consisting of pentane, hexane, octane, iso-octane, and cyclohexane.
5. The method of claim 1 wherein the solvent is selected from the group consisting of methanol, ethanol, isopropyl alcohol, acetonitrile, and acetic acid.

6. The method of claim 1 wherein the salt is an inorganic salt selected from the group consisting of alkali metals and alkali earth metals.

7. The method of claim 1 wherein the salt is an inorganic salt selected from the group consisting of sodium chloride, potassium chloride, and magnesium sulfate.

8. The method of claim 1 wherein the solvent used to form the extract is methanol, the water-immiscible organic solvent is petroleum ether, and the salt is sodium chloride.

9. The method of claim 1 wherein the solvent used to form the extract is a water-immiscible organic solvent.

10. The method of claim 9 wherein the material of animal or vegetable origin, or vitamin product is selected from the group consisting of poultry meal, meat and bone meal, fish meal, poultry fat, animal fat, 5 tallow, lard, yellow grease made from animal or vegetable oils and fats, by-product meals, vitamins and vitamin premixes, and DAF sludge.

11. The method of claim 10 wherein the water-immiscible organic solvent is selected from the group consisting of petroleum ether, toluene, chloroform, and alkanes.

12. The method of claim 11 wherein the salt is an inorganic salt selected from the group consisting of sodium chloride, potassium chloride, and magnesium sulfate.

13. The method of claim 12 wherein the water-immiscible organic solvent is petroleum ether and the salt is sodium chloride.

14. A method for approximating the concentration of ethoxyquin in a material of animal or vegetable origin, or vitamin product comprising:

5 combining a sample of the material with a polar solvent to form a first extraction mixture comprising a solid residue fraction and a liquid fraction containing the polar solvent;

10 forming a second extraction mixture which comprises a salt, water, water-immiscible organic solvent, and the polar solvent from the first extraction mixture;

separating the water-immiscible organic solvent from the polar solvent in a single extraction step; and

15 determining the concentration of ethoxyquin in the separated water-immiscible organic solvent using a fluorometer.

15. The method of claim 14 wherein the material of animal or vegetable origin, or vitamin product is selected from the group consisting of poultry meal, meat and bone meal, fish meal, poultry fat, animal fat, 5 tallow, lard, yellow grease made from animal or vegetable oils and fats, by-product meals, vitamins and vitamin premixes, and DAF sludge.

16. The method of claim 15 wherein the water-immiscible organic solvent is selected from the group consisting of petroleum ether, toluene, chloroform, and alkanes.

17. The method of claim 16 wherein the polar solvent is selected from the group consisting of methanol, ethanol, isopropyl alcohol, acetonitrile, and acetic acid.

18. The method of claim 17 wherein the salt is an inorganic salt selected from the group consisting of sodium chloride, potassium chloride, and magnesium sulfate.

19. The method of claim 18 wherein the solvent is methanol, the water-immiscible organic solvent is petroleum ether, and the salt is sodium chloride.

20. A method for approximating the concentration of ethoxyquin in a material of animal or vegetable origin, or vitamin product comprising:

5 combining a sample of the material with a solvent to form a first extraction mixture comprising a solid residue fraction and a liquid fraction containing the solvent;

10 forming a second extraction mixture which comprises a salt, water, a water-immiscible organic solvent, and the solvent from the first extraction mixture, the second extraction mixture having an absence of a solvent from extraction mixtures containing the solid residue other than the first extraction mixture;

15 separating water-immiscible organic solvent from the solvent; and

determining the concentration of ethoxyquin in the separated water-immiscible organic solvent using a fluorometer.

21. The method of claim 20 wherein the water-immiscible organic solvent is separated from the solvent in a single extraction step.

22. The method of claim 20 wherein the material of animal or vegetable origin, or vitamin product is selected from the group consisting of poultry meal, meat, and bone meal, fish meal, poultry fat, animal fat, 5 tallow, lard, yellow grease made from animal or vegetable oils and fats, by-product meals, vitamins and vitamin premixes, and DAF sludge.

23. The method of claim 22 wherein the water-immiscible organic solvent is selected from the group consisting of petroleum ether, toluene, chloroform, and alkanes.

24. The method of claim 23 wherein the solvent is selected from the group consisting of methanol, ethanol, isopropyl alcohol, acetonitrile, and acetic acid.

25. A method for approximating the concentration of ethoxyquin in a material of animal or vegetable origin, or vitamin product comprising:

combining a sample of the material with a solvent to 5 form a first extraction mixture comprising a solid residue fraction and a liquid fraction containing the solvent, the first extraction mixture being formed without grinding the material to a desired size;

forming a second extraction mixture which comprises 10 a salt, water, a water-immiscible organic solvent, and solvent from the first extraction mixture;

separating the water-immiscible organic solvent from the solvent; and

determining the concentration of ethoxyquin in the
15 separated water-immiscible organic solvent using a
fluorometer.

26. The method of claim 25 wherein the material of animal or vegetable origin, or vitamin product is selected from the group consisting of poultry meal, meat and bone meal, fish meal, poultry fat, animal fat, 5 tallow, lard, yellow grease made from animal or vegetable oils and fats, by-product meals, vitamins and vitamin premixes, and DAF sludge.

27. The method of claim 26 wherein the water-immiscible organic solvent is selected from the group consisting of petroleum ether, toluene, chloroform, and alkanes.

28. The method of claim 27 wherein the solvent is selected from the group consisting of methanol, ethanol, isopropyl alcohol, acetonitrile, and acetic acid.

29. The method of claim 28 wherein the salt is an inorganic salt selected from the group consisting of sodium chloride, potassium chloride, and magnesium sulfate.

30. The method of claim 29 wherein the solvent is methanol, the water-immiscible organic solvent is petroleum ether, and the salt is sodium chloride.

31. A method for approximating the concentration of ethoxyquin in a material of animal or vegetable origin, or vitamin product comprising:
5 combining a sample of the material with a solvent to form a first extraction mixture comprising a solid

residue fraction and a liquid fraction containing the solvent, the first extraction mixture being formed without measuring the mass of the sample within 0.1 grams;

10 forming a second extraction mixture which comprises a salt, water, a water-immiscible organic solvent, and solvent from the first extraction mixture;

separating the water-immiscible organic solvent from the solvent; and

15 determining the concentration of ethoxyquin in the separated water-immiscible organic solvent using a fluorometer.

32. The method of claim 31 wherein the sample is measured volumetrically.

33. The method of claim 31 wherein the second extraction mixture has an absence of a solvent from extraction mixtures containing the solid residue other than the first extraction mixture.

34. The method of claim 33 wherein the water-immiscible organic solvent is separated from the solvent in a single extraction step.

35. The method of claim 34 wherein the first extraction mixture being formed without grinding the material to a desired size.

36. The method of claim 35 wherein the material of animal or vegetable origin, or vitamin product is selected from the group consisting of poultry meal, meat and bone meal, fish meal, poultry fat, animal fat,
5 tallow, lard, yellow grease made from animal or vegetable

oils and fats, by-product meals, vitamins and vitamin premixes, and DAF sludge.

37. The method of claim 36 wherein the water-immiscible organic solvent is selected from the group consisting of petroleum ether, toluene, chloroform, and alkanes.

38. The method of claim 37 wherein the solvent is selected from the group consisting of methanol, ethanol, isopropyl alcohol, acetonitrile, and acetic acid.

39. The method of claim 38 wherein the salt is an inorganic salt selected from the group consisting of sodium chloride, potassium chloride, and magnesium sulfate.

40. The method of claim 31 wherein the solvent is methanol, the water-immiscible organic solvent is petroleum ether, and the salt is sodium chloride.

41. A method for approximating the concentration of ethoxyquin in a material of animal or vegetable origin, or vitamin product comprising:

5 forming an extraction mixture which comprises a sample of the material, a salt, water, and a water-immiscible organic solvent;

separating the water-immiscible organic solvent from the extraction mixture; and

10 determining the concentration of ethoxyquin in the separated water-immiscible organic solvent using a fluorometer.

42. The method of claim 41 wherein the material of animal or vegetable origin, or vitamin product is

selected from the group consisting of poultry meal, meat and bone meal, fish meal, poultry fat, animal fat,
5 tallow, lard, yellow grease made from animal or vegetable oils and fats, by-product meals, vitamins and vitamin premixes, and DAF sludge.

43. The method of claim 42 wherein the water-immiscible organic solvent is selected from the group consisting of petroleum ether, toluene, chloroform, and alkanes.

44. The method of claim 43 wherein the salt is an inorganic salt selected from the group consisting of sodium chloride, potassium chloride, and magnesium sulfate.

45. The method of claim 44 wherein the water-immiscible organic solvent is petroleum ether and the salt is sodium chloride.

INTERNATIONAL SEARCH REPORT

International application No
PCT/US 00/16286

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N21/64

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, PAJ, WPI Data, INSPEC, COMPENDEX, IBM-TDB, BIOSIS, FSTA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	S.C. WITT ET AL.: "Simplified method for the determination of ethoxyquin in alfalfa products and mixed feeds" JOURNAL OF THE ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS, vol. 56, no. 1, 1973, pages 167-170, XP000961315 abstract page 167, right-hand column, line 1 - line 5	1,3-9, 14,20, 21,25, 31,41
A	page 167, right-hand column, last paragraph -page 168, left-hand column, line 31 ---	11,16, 17,23, 24,27, 28,37, 38,43
		-/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

Date of the actual completion of the international search

13 November 2000

Date of mailing of the international search report

21/11/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patendaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040. Tx. 31 651 epo nl.
Fax: (+31-70) 340-3016

Authorized officer

Thomas, R.M.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/16286

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	G.A. PERFETTI ET AL.: "Reverse phase high pressure liquid chromatography and fluorescence detection of ethoxyquin in milk" JOURNAL OF THE ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS, vol. 66, no. 5, September 1983 (1983-09), pages 1143-1147, XP000961366 page 1144, right-hand column, line 3 - line 41 page 1144, right-hand column, last paragraph -page 1145, left-hand column, line 18 page 1145, left-hand column, line 34 - line 35	1,3-9, 14,20, 21,25, 31,41 11-13, 16-19, 23,24, 27-30, 37-40, 42-45
A	---	
A	H.R. WEILENMANN ET AL.: "Quantitative Bestimmung von Santoquin an Äpfeln mit Hilfe der Fluoreszenz-Spektrophotometrie und Dünnschicht-Chromatographie" LEBENSMITTEL-WISSENSCHAFT UND TECHNOLOGIE, vol. 5, no. 3, 1972, pages 106-107, XP000957936 page 106, right-hand column, last paragraph -page 107, left-hand column, line 10	1,3,4,6, 9,11,14, 16,20, 25,27, 31,37, 41,43
A	---	
A	K. FUJINUMA ET AL.: "Determination of ethoxyquin in spices" JOURNAL OF THE FOOD HYGIENIC SOCIETY OF JAPAN, vol. 23, no. 1, 1982, pages 67-72, XP000961312 abstract	1,3,4,9, 11,14, 16,20, 25,27, 31,37, 41,43
A	---	
A	W.L. DUNKLEY ET AL.: "Compounds in milk accompanying feeding of ethoxyquin" JOURNAL OF DAIRY SCIENCE, vol. 51, no. 8, 1968, pages 1215-1218, XP000953366 page 1215, right-hand column, paragraph 1 - paragraph 2	1,3,4,9, 11,14, 16,20, 25,27, 31,37, 41,43
	---	-/-

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 00/16286

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J.U. SKAARE ET AL.: "Ethoxyquin (EMQ) residues in atlantic salmon measured by fluorimetry and gas chromatography (GLC)" NORDISK VETERINAERMEDICIN, vol. 29, no. 4/5, 1977, pages 232-236, XP000961411 page 233, left-hand column, line 27 - line 38 -----	1,14,20, 25,31,41